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CONTRACT No. DAJB17-67-C-0097

BIOLOGIC REACTIONS OF CELLULAR ANTIBODIES WITH SPECIAL REFERENCE TO THEIR IMMUNO-PATHOLOGICAL AND IMMUNO-CHEMICAL PROPERTIES

by

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of the First Department,
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Kumamoto, Japan

August 1967

U. S. ARMY RESEARCH AND DEVELOPMENT GROUP
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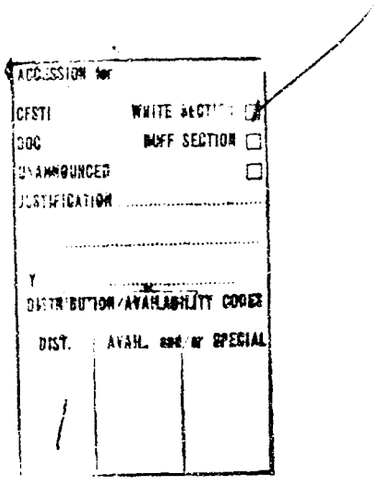

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Abstract

As previously described, the cells, cultivated from the omentum of BSA-sensitized rabbits and grown in a special culture medium, were shown to contain antibody to the antigen, as detected by the fluorescent antibody technique. The cells were possibly originated in the reticulum cells and termed "omentum cells"; and they seemed useful for the study of cell-bound antibody.

The cell-bound antibody was separated from the cell extracts or culture fluid medium and partially purified by fractionation with ammonium sulfate followed by gel filtration with Sephadex G-200 or by sucrose density gradient centrifugation; and the presence of antibodies of 7S and 19S classes was confirmed.

The cell-bound 19S antibody was found to have a characteristic ability to sensitize the homologous skin (rabbit skin) and to cause intensively the passive cutaneous Arthus reaction and reversed passive Arthus reaction in rabbit skin. Such biologic activity was found apparently less marked or negligible when the

cell-bound 7S antibody or serum 7S and 19S antibody were assayed similarly in the rabbit skin. Further purification of cell-bound 19S antibody is being advanced.

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The Study of Cell-Bound Antibody: Further Demonstration of Antibody in Omentum Cells in Culture

In previous studies, it was ascertained that the cells, cultivated from the omentum of bovine serum albumin-sensitized rabbits and grown in our special culture medium, contained antibodies to bovine serum albumin, as detected by fluorescent antibody technique; and they showed characteristic morphologic changes affecting the cell membrane and ground cytoplasm when the antigen was introduced into the culture (1 - 5). The antigen-induced morphologic changes in the cells were associated with some biochemical changes; for instance, activation and release of a specific sulfhydryl-dependent protease (termed Arthus protease) and its inhibitor of peptide nature (6, 7). The activation of Arthus protease in the cells seemed correlated with production and release of histamine and specific vascular permeability factor of peptide nature (termed Arthus permeability factor) (8, 9). The cells were conventionally called "omentum cells". Arthus protease and its inhibitor were recovered in the euglobulin fraction prepared from the Arthus skin lesions, and Arthus permeability factor in the pseudoglobulin fraction prepared; and all these substances were highly purified; and their

significant role in the Arthus skin lesions were confirmed (10 - 12).

The present paper describes further confirmation of antibodies of 7S and 19S classes in the omentum cells of such biologic significance in culture.

Materials and Methods

Procedures of Sensitization:

Male albino rabbits (1.8 - 2.0 kg) bred in our laboratory were used. 0.5 ml. of bovine serum albumin (BSA, recrystallized twice, 20 mg. per ml. in saline; Armour), mixed with an equal volume of Freund's complete adjuvant, was injected intramuscularly in the skin of the back of the trunk of the animals. Ten or twenty injections (once per week) were given. On the 7th day after the last injection, each animal's

serum antibody was determined by an indirect hemagglutination test (13). Only animals with titers of over 30×2^{15} were used.

Cultivation of Omentum Cells:

In earlier studies (14), mononuclear cells (histiocytes) of the peritoneal fluid of sensitized rabbits were selected because of their high antibody content. In the present work, "omentum cells" from the omentum pieces were used because of their easier cultivation and better growth.

Details of the methods for omentum cell cultivation have been given in previous papers (2, 15, 25). The explants, 1 mm. in diameter, were prepared from the omentum of sensitized or nonsensitized animals. Fluid medium was composed of horse serum, 5 per cent lactalbumin hydrolysate (Nutritional Biochemicals Corporation) in Gey's buffered solution, and Gey's fluid in a ratio of 1:1:8, and adjusted to pH 7.2.

After 3 - 4 days, almost every cell in each culture was omentum cell. Observation of cultured cells was

made with a Tiyoda Phase contrast microscope. The numbers of cells were counted by the method of Katsuda (16) with 0.1 M citric acid containing crystal violet in a concentration of 0.02 per cent. The cell numbers were generally 30,000 (± 3,000) per culture. Only cultures showing such similar growth conditions were used in the present work.

Preparation of Cell Extracts:

Shortly after sufficient washing (three times) with buffered saline, the cells (3×10^6 in general) were suspended in 3 - 5 ml. buffered saline (0.15 M, pH 7.8). After homogenizing for 5 - 7 min. in the cold, the cells were extracted with 3 ml. buffered saline for 16 hrs. After centrifuge at 30,000 r.p.m. for 10 min., the supernatant fluid was used as the cell extracts.

Preparation of Euglobulin Fraction from Cell Extracts:

From the cell extracts (5 - 8 ml), euglobulin

fraction was prepared with sodium sulfate at 18 per cent saturation (17), and dialysed against phosphate buffer (0.01 M, pH 8.0). Euglobulin fraction was also prepared from culture fluid medium (80 ml) with ammonium sulfate at 35 per cent saturation.

Experimental Results

Detailed morphology of omentum cells in culture has been given in a previous paper (2, 15). There were observed spindle-and reticular type cells (Fig. 1). No other cell types were found in each culture. Since the spindle-type cells were often observed to have changed to the reticular-type cells under the phase contrast microscope, it seemed reasonable that these cells were essentially identical and came from the same precursor cells of the omentum, perhaps reticulum cells.

1. Demonstration of Antibodies in Cell Extract and its Euglobulin Fraction:

The antibody titers of test samples were measured by the indirect sheep red cell agglutination method of Boyden (13).

As summarized in Table 1, the antibody to BSA was first detected in the cell extracts as well as culture fluid medium, indicating the extracellular release of the antibody during the cultivation. The antibody was similarly concentrated in the euglobulin fractions prepared from the two sources. No antibody was detected with all the samples prepared from omentum cells of nonsensitized normal rabbits.

2. Demonstration of Antibodies of 7S and 19S Classes in Cell Extract and its Euglobulin Fraction: Elution on Sephadex G-200:

After dialysing against Tris HCl buffer (0.1 M, pH 8.0), 2 ml. of euglobulin fraction described above

was eluted through a column (2 x 100 cm.) of Sephadex G-200 (Pharmacia) (18). The rate of flow was 30 ml. per hour and 4-g effluent fractions were collected. The concentration of euglobulin fraction from cell extracts was 7.1 mg per ml. and that of the protein fraction from culture fluid medium was 28.7 - 33.4 mg. per ml. Concentration of protein fractions was made by dialysis against 20 per cent polyvinyl pyrolidone. The same type of experiments was carried out with euglobulin fractions of omentum cells of nonsensitized normal rabbits.

As illustrated in Fig. 1, the presence of antibody was revealed in the chromatographic patterns associated with 7S and 19S fractions, though the hemagglutination titers were in particular very low in the euglobulin fraction from the cell extracts. Higher content of antibody in the culture fluid medium indicated that the extracellular release of antibody occurred during the cultivation of omentum cells.

3. Demonstration of Antibodies of 7S and 19S Classes in Cell Extract and its Euglobulin Fraction: Analysis by Sucrose Density Gradient:

Euglobulin fraction from culture fluid medium was used because of its higher antibody content. The protein fraction was dialysed against 5 per cent sodium chloride for 16 hrs. 0.5 ml. protein fraction (at concentration of 32.0 mg. per ml.) received a sucrose density gradient centrifugation (19). Concentrations of proteins were recorded as the absorbancy, E, at 280 mμ.

As illustrated in Fig. 2, the presence of antibody was demonstrated in the patterns associated with 7S and 19S fractions. The observations closely resembled those on euglobulin fractions after elution on Sephadex G-200. Hemagglutination titers of 7S fractions were, as a rule, much higher than those of 19S fractions.

Discussion

As previously described (1, 2, 15), our "omentum cells" in culture were clearly different from plasma cells; the cells seemed to originate from the reticulum cells in the omentum tissue. Immuno-histochemical examination of the tissue not subjected to the cultivation revealed the presence of specific fluorescence in the "omentum cells" and plasma cells (3), but plasma cells were found to disappeared, due to their degenerative change, very shortly after cultivation. The detection of the antibody in the cell extracts indicated that the antibody was undoubtedly associated with the omentum cells, but not with the plasma cells, because the cells extracts were prepared from cultures in which plasma cells were not observed.

It was very interesting to note that the omentum cells contained antibodies of 7S and 19S classes. These antibodies were in part released from omentum cells during the cultivation, as revealed in the culture fluid medium; and it was suggested that the antibody

might be detected in the body fluid. Recently, Juhasz and Rose (20) and Juhasz and Richter (21) demonstrated the presence of 7S and 19S type antibodies in a fluid medium of cultures of lymph nodes from rat albumin-sensitized rabbits. The cellular origin of these antibodies was difficult to determine, because of complicated cell population of the lymph node in culture.

The cytophilic antibody of Boyden and Sorkin (22, 23), behaving as a 7S globulin, failed to sensitize gunia pig skin in passive cutaneous anaphylaxis (P.C.A.). The 7S type antibody of omentum cells showed no P.C.A. in rabbit skin, but the 19S type antibody was very active in inducing the P.C.A. (24), indicating its specific biologic function. The biologic properties of the cell 19S antibody will be described in a separate paper.

Summary

The cells, which were cultivated from the omentum of BSA-sensitized rabbits and grown in a special culture

medium, were shown to contain antibodies of 7S and 19S classes; the separation of each type of antibodies was performed by gel filtration (with Sephadex G-200) or sucrose density gradient centrifugation of cell extracts and culture fluid medium. The cells seemed to originate from the reticulum cells of the omentum and were termed "omentum cells". They seemed very useful for the study of cell-bound antibody.

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APPENDIX "A"

A n t i b o d y t i t e r s

Group of rabbits	Cell exp.	Eugl.cell exp.	Cult. med.	Eugl.cul. med.	Serum
Sensitized A	2^5	2^6	2^7	2^8	30×2^{15}
Sensitized B	2^7	2^8	2^8	2^{10}	30×2^{17}
Sensitized C	2^9	n.t.	2^{10}	2^{11}	30×2^{17}
Nonsensitized D	0	n.t.	0	0	0

Table 1. Demonstration of antibody in cell extract or culture fluid medium and in their euglobulin fractions. Assayed by indirect hemagglutination test.
ext.: extract. eugl.: euglobulin. Cult.med.: culture medium.
Cell extract: 4.2 mg/ml.; its euglobulin: 8.5 mg/ml.
Culture medium: 20 mg/ml; its euglobulin: 31 mg/ml.

APPENDIX "B"

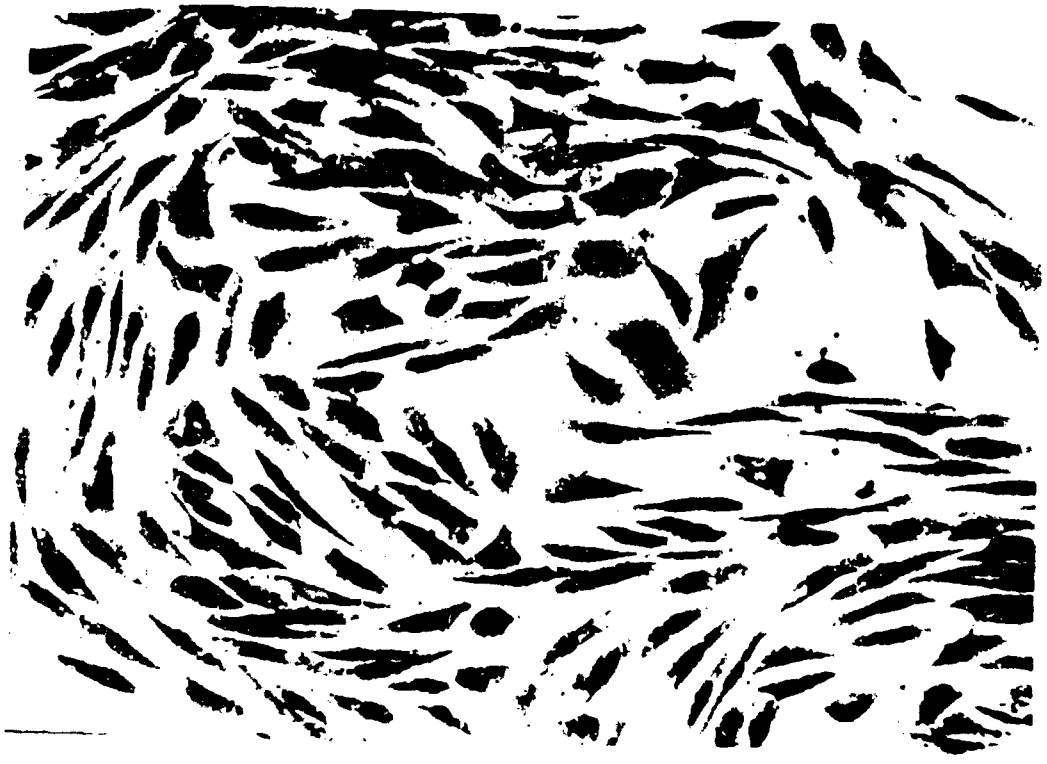

Fig. 1. "Omentum cells" in 4 day old culture. There were observed spindle-and reticular-type cells; no other cell types were found. They were essentially identical and perhaps came from the same precursor cells of the omentum, the reticulum cells. Detailed morphology has been previously given (2, 15, 25).

APPENDIX "C"

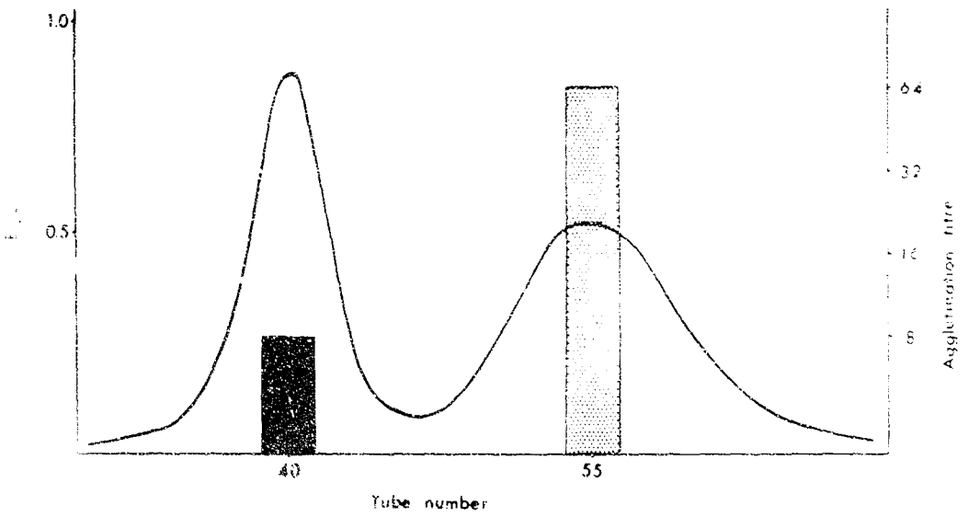

Fig. 2. Antibodies of Culture fluid Medium on Sephadex G-200. Euglobulin, prepared from culture fluid medium, was eluted through Sephadex G-200 column. 19S antibody fraction in the first column. 7S antibody fraction in the second column.

APPENDIX "D"

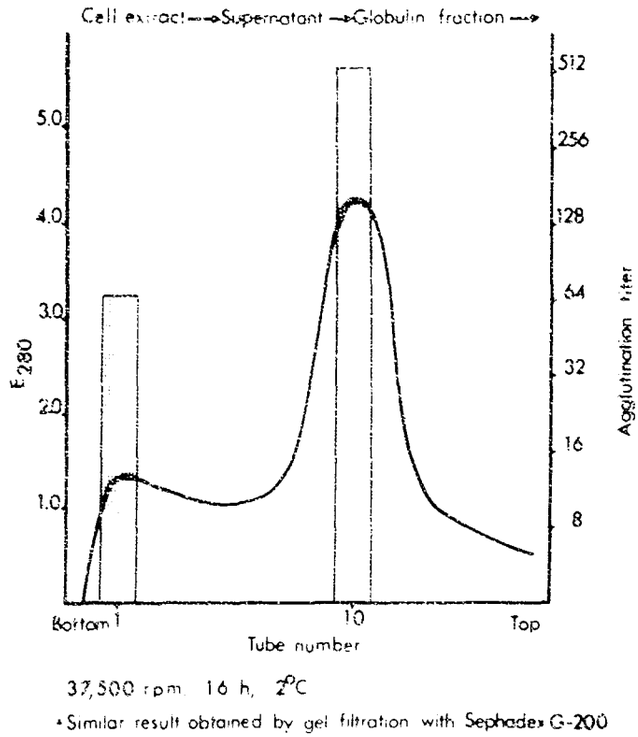

Fig. 3. Antibodies of Cell Extracts on Sucrose Density Gradient Centrifugation. Buglobulin, prepared from cell extracts, received a sucrose density gradient centrifugation (375,000 r.p.m., 16 hrs. 2°C). 7S ~~19S~~ antibody fraction in the first column. ~~19S~~ antibody fraction in the second column.

The Study of Cell-Bound Antibody: The Biologic Properties of Antibodies in Omentum Cells in Culture

In previous studies (1 - 4), we demonstrated the presence of antibodies of 7S and 19S classes in the cells (termed "omentum cells") which were cultivated from the omentum of BSA-sensitized rabbits and grown in a special culture medium; and these antibodies were extracted from the cultured cells and partially purified.

The biochemical changes by the antigen in the cultured cells were characterized by the rapid activation of a specific SH-dependent protease (termed "Arthus protease") which was associated with the release of histamine and Arthus permeability factor of peptide nature (5 - 9). The question of which antibody in the cells was responsible for the biochemical changes described above has not yet been clarified.

The present report deals with the particularly significant action of 19S type antibody extracted from the omentum cells, as shown in form of skin fixability in a homologous animal.

Materials and Methods

Separation of Antibodies:

Details of procedures for sensitization of rabbits with BSA, cultivation of omentum cells and extraction of antibodies all have been given in previous papers (2, 4, 10).

From the fluid medium of 3-4 day old cultures, euglobulin fraction was prepared with ammonium sulfate at 35 per cent saturation, and suspended in Tris-HCl buffer (0.1 M, pH 8.0) at a concentration of 40 mg. per ml. Four ml. of the protein fraction was passed through a column (4 x 100 cm) of Sephadex G-200 (Pharmacia) (11). The flow rate was 30 ml. per hour and 6-g effluent fractions were collected; and effluent fractions were analysed, particularly by ultraviolet light absorption at 280 mμ; and there were obtained two chromatographic components. The first component contained 19S class antibody and the second component contained 7S class antibody when assayed by an indirect sheep red cell

agglutination test (12).

Separation of 19S class antibody fraction from rabbit's antisera was performed following the method of Vaerman et al (13) utilizing sucrose density gradient centrifugation or Sephadex G-200 filtration. Separation of 7S class antibody fraction from rabbit's antisera was made by the method of Kapusta and Halberstam (14); and the protein fraction after chromatography with DEAE-cellulose was used. Purity of these antibody fractions was examined immuno-electrophoretically (15).

Induction of Passive Cutaneous Anaphylaxis (P.C.A.):

This was essentially performed following the method of Ovary (16). Before the test on rabbit skin, all the antibody fractions were dialysed against physiological saline. 0.1 ml. of each antibody fraction was intradermally injected on the clipped flanks of rabbits (2 - 2.2 kg.). At 5 and 30 minute, 3, 6, 12, 48 and 72 hour intervals after injections of antibody fractions, BSA (12.5 mg. per animal) was intravenously administrated simultaneously with pontamine blue (60 mg. per

kg), and 30 min. later the animals were sacrificed. Hemagglutination titers of each antibody fraction injected were 2^6 in general. The intensity of passive cutaneous Arthus reaction was quantitatively expressed in terms of exuded dye extractable from the skin sites in rabbits (17).

In control experiments, 7S or 19S globulin fraction, similarly prepared from nonsensitized normal omentum cells, was used instead of antibody fractions.

Induction of Reversed Passive Arthus Reaction:

At various intervals after intravenous injection of BSA (12.5 mg. per ml.), 0.1 ml. of antibody fraction (2^6 in the indirect hemagglutinin titers) was intradermally injected in the clipped flanks and pontamine blue (60 mg. per kg) was injected intravenously; and 30 min. later exuded dye was extracted from the injected skin sites (18). The intensity of reversed passive Arthus reaction was shown by the amount (μg) of the extracted dye.

In control experiments, 7S or 19S globulin fractions,

similarly prepared from nonsensitized normal omentum cells, was utilized instead of antibody fractions.

Experimental Results

I. Passive Cutaneous Anaphylaxis by Different Antibodies from BSA-Sensitized Rabbits:

The intensity of passive cutaneous Arthus reaction in a homologous animal (rabbit) was shown by the amount of dye extracted from the skin sites.

As summarized in Table I, 19S antibody fraction, prepared from cultured omentum cells, was very active in inducing passive cutaneous Arthus reaction in the rabbit skin, but the effects of other antibody fractions from cultured cells or from antisera all were only mild.

Negative results were obtained with 19S globulin fraction, isolated from nonsensitized normal omentum cells and prepared at the same concentration. It was

thus reasonably indicated that the 19S class antibody of sensitized omentum cells had a strong ability to cause passive cutaneous Arthus reaction in a homologous animal, i.e., rabbit.

Furthermore, it was confirmed that the effects of sensitization of rabbit skin with the cellular 19S antibody for induction of passive cutaneous Arthus reaction became apparent at one hour after intradermal injection of the antibody, reached its peak at three hours after the injection and declined thereafter. Such relatively rapid sensitization of the rabbit skin with the cellular 19S antibody seemed to characterize the antibody.

II. Reversed Passive Arthus Reaction by Different Antibodies from BSA-Sensitized Rabbits:

The reversed passive Arthus reaction in the rabbit skin, induced by different antibodies of BSA-sensitized rabbits, was compared in the intensity of vascular permeability changes in the reaction sites. The intensity of the permeability changes was shown by the amount of

the extracted dye.

As illustrated in Fig. 1, the permeability change due to the cellular 19S antibody clearly appeared most active; and the change was diphasic — immediate and delayed. The immediate response was clearly transient and declined within 10 min., but the delayed response was far more intense and prolonged; the response reached its peak in about 2 hrs., declined gradually therafter, and disappeared in about 3-4 hrs.

On the other hand, the permeability change by the cellular 7S antibody was less marked; the immediate response was almost negative. Similar but less marked permeability change was also recognized at the skin sites injected with the serum 7S antibody; the immediate response was clearly negative. The serum 19S antibody failed to cause any vascular permeability change in the rabbit skin. Furthermore, negative results were obtained with 7S and 19S globulins from nonsensitized normal omentum cells or from the sera of nonsensitized normal rabbits.

Discussion

The cells ("omentum cells"), cultivated from the omentum of BSA-sensitized rabbits and grown in a special culture medium, were shown to contain anti-BSA antibodies of 7S and 19S classes (1 - 4). The observations just presented indicated the characteristic properties of the cell-bound 19S antibody.

When assayed for inducing passive cutaneous Arthus reaction in the skin of homologous animal (rabbit skin), the effects of the cell-bound 19S antibody were apparently striking; and the sensitization of the rabbit skin with the antibody seemed to reach its peak at about 3 hrs. after the intradermal injection (Table 1). In contrast to the delayed sensitization of rabbit skin with serum antibody (IgA) of Zweifler and Becker (19) and Onoue et. al. (20), such rapid sensitization with our cell-bound antibody seemed very important.

Furthermore, when assayed for inducing the vascular permeability changes in the reversed passive Arthus reaction in the rabbit skin, the effects of the

cell-bound 19S antibody were also very active; and the permeability changes appeared diphasic; the immediate and delayed response. The time course of the permeability changes closely resembled those seen in an active Arthus reaction (18), indicating the biologic significance of the cell-bound 19S antibody.

It was very interesting to note that the immediate vascular response was not induced by the cell-bound 7S antibody or the serum 7S and 19S antibody; such negative observations with these antibodies appeared to be reasonable because the immediate vascular response essentially seemed identical with that in the passive cutaneous Arthus reaction. These antibodies also failed to cause the passive cutaneous Arthus reaction in the rabbit skin (Table 1).

As described above, the cell-bound 19S antibody was shown to have a previously undescribed property on characteristic sensitization of the homologous skin. Further study of purification of the cell-bound antibody is being performed in this laboratory.

Summary

The cells, grown in culture from the omentum of BSA-sensitized rabbits, contained antibodies of 7S and 19S classes. The cells (termed "omentum cells") were possibly originated in the reticulum cells of the omentum. The cell-bound 19S class antibody had a characteristic ability to sensitize the homologous (rabbit) skin and its biologic significance was briefly discussed.

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APPENDIX "A"

Samples used	Latent Period: minutes		hours			
	30	60	2	3	12	48
	Extracted dye (ug)					
Cell 19S*	2.1	6.8	15.0	19.7	2.5	1.5
Cell 7S*	1.5	3.2	4.0	3.9	1.4	2.0
Serum 19S*		1.9		1.9	1.6	1.9
Serum 7S*		3.5		3.6	2.3	1.6
Cell 19S gl.**		2.2		2.5		
Cell 7S gl.**		1.7		1.6		

Table 1. Passive cutaneous Arthus reaction by different antibodies. The intensity is shown by the amount (µg) of extracted dye. Latent Periods mean the time intervals after intradermal antibody.
Cell 7S, 19S: extracted from sensitized cells.
Cell 7S, 19S gl.: extracted from nonsensitized normal cells. Serum 7S, 19S: isolated from antisera of sensitized rabbits. The values represent the average of several determinations.

* tested at the same hemagglutination titers of each antibody fraction (2^6).

** tested at the same concentration as that of the cell 7S and 19S antibody fractions (0.3 mg/ml.).

APPENDIX "B"

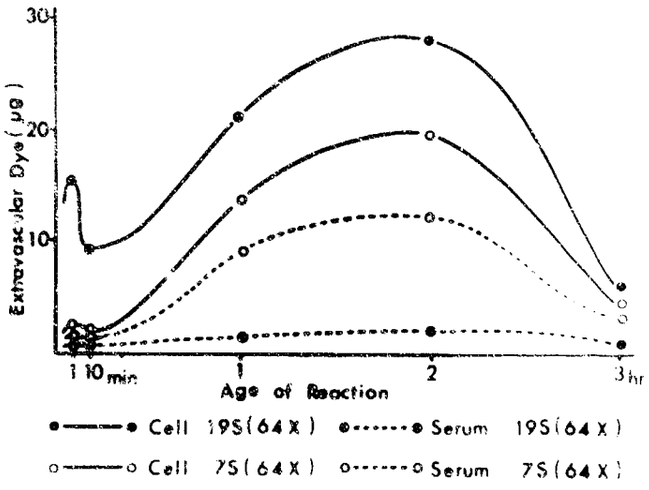

Fig. 1. Vascular Permeability Changes in Reversed Passive Arthus Reaction by Cellular and Serum Antibodies. Each sample was prepared to give the same indirect hemagglutination titers before test. The intensity of the permeability change is shown by the amount (µg) of extracted dye.

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2b. GROUP

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13. ABSTRACT

As previously described, the cells, cultivated from the omentum of BSA-sensitized rabbits and grown in a special culture medium, were shown to contain antibody to the antigen, as detected by the fluorescent antibody technique. The cells were possibly originated in the reticulum cells and termed "omentum cells"; and they seemed useful for the study of cell-bound antibody.

The cell-bound antibody was separated from the cell extracts or culture fluid medium and partially purified by fractionation with ammonium sulfate followed by gel filtration with Sephadex G-200 or by sucrose density gradient centrifugation; and the presence of antibodies of 7S and 19S classes was confirmed.

The cell-bound 19S antibody was found to have a characteristic ability to sensitize the homologous skin (rabbit skin) and to cause intensively the passive cutaneous Arthus reaction and reversed passive Arthus reaction in rabbit skin. Such biologic activity was found apparently less marked or negligible when the cell-bound 7S antibody or serum 7S and 19S antibody were assayed similarly in the rabbit skin. Further purification of cell-bound 19S antibody is being advanced. (Author)

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